Culture-Independent Characterization of a Novel, Uncultivated Magnetotactic Member of the *Nitrospirae* Phylum

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Summary

A magnetotactic bacterium, designated strain LO-1, of the *Nitrospirae* phylum was detected and concentrated from a number of freshwater and slightly brackish aquatic environments in southern Nevada. The closest phylogenetic relative to LO-1 is *Candidatus* Magnetobacterium bavaricum based on a 91.2% identity in their 16S rRNA gene sequence. Chemical and cell profiles of a microcosm containing water and sediment show that cells of strain LO-1 are confined to the oxic–anoxic interface and the upper regions of the anaerobic zone which in this case, occurred in the sediment. This microorganism is relatively large, ovoid in morphology and usually biomineralizes three braid-like bundles of multiple chains of bullet-shaped magnetosomes that appeared to be enclosed in a magnetosome membrane. Cells of LO-1 had an unusual three-layered unit membrane cell wall and contained several types of inclusions, some of which are sulfur-rich. Strain LO-1 is motile by means of a single bundle of sheathed flagella and exhibits the typical 'wobbling' motility and helical swimming ('flight') path of the magnetotactic cocci. This study and reports from others suggest that LO-1-like organisms are widespread in sediments of freshwater to brackish natural aquatic environments.

Introduction

Magnetotactic bacteria represent a diverse group of prokaryotes that biomineralize intracellular singlemagnetic-domain crystals of the iron oxide magnetite (Fe3O4) and/or the iron sulfide greigite (Fe3S4) (Bazylinski and Frankel, 2004). These crystals, together with the membrane that envelops them, are referred to as magnetosomes. Magnetosomes impart a permanent magnetic dipole moment to the cell causing it to align along the Earth's geomagnetic field lines like a miniature compass needle (Frankel *et al*., 1997). The prevailing theory of the function of magnetosomes is that these organelles help microaerophilic or anaerobic magnetotactic bacterial cells locate and maintain an optimal position in vertical chemical gradients (e.g. O2 gradients) in chemically stratified environments by increasing the efficiency of chemotaxis (Frankel *et al*., 1997). Because there are a relatively small number of strains of magnetotactic bacteria in axenic culture, environmental studies of uncultured species are important not only for the development of cultivation techniques but also for information about magnetotactic prokaryotes in general.

Phylogenetically, known magnetotactic bacteria are members of several classes of the *Proteobacteria* phylum including the *Alpha*-, *Gamma*-and *Deltaproteobacteria*, and the *Nitrospirae* phylum (Amann *et al*., 2006). The *Nitrospirae* phylum is a group of diverse Gram-negative bacteria that currently contains only three genera that have representatives in culture: *Nitrospira*, *Leptospirillum* and *Thermodesulfovibrio*. Species of each genus have very different physiologies, and phenotypic links between the genera are not obvious. For example, *Nitrospira* species are aerobes that oxidize nitrite (Watson *et al*., 1986; Ehrich *et al*., 1995) while those of *Leptospirillum* genus are aerobic, acidophilic iron-oxidizing bacteria (Hippe, 2000). The third genus, *Thermodesulfovibrio*,isa group of thermophilic, anaerobic sulfate-reducing bacteria (Henry *et al*., 1994; Sonne-Hansen and Ahring, 1999; Haouari *et al*., 2008; Sekiguchi *et al*., 2008). Thus, due to the relatively small amount of information regarding organisms of the *Nitrospirae* phylum, it is difficult to ascertain the potential of this group in biogeochemical cycling.

There are many reports of uncultured *Nitrospirae* in environmental diversity studies using culture-independent techniques. In most cases, other than the 16S rRNA gene sequence, nothing is really known regarding these organisms. This uncultured group includes some magnetotactic bacteria and three described morphotypes have been found to be phylogenetically affiliated with the *Nitrospirae* phylum thus far (Spring *et al*., 1993; Flies *et al*., 2005; Lefèvre *et al*., 2010). None have been cultured and none are closely related phylogenetically to cultured members of the group and little is known regarding their physiology except what has been inferred from their ecology. Interestingly, cells of all three morphotypes biomineralize bullet-shaped magnetite crystals in their magnetosomes (Lefèvre *et al*., 2010). The large rod, *Candidatus* Magnetobacterium bavaricum, is the most studied of the three and was first discovered in sediment samples from Lake Chiemsee and Lake Ammersee in southern Germany (Vali *et al*., 1987; Petersen *et al*., 1989). Cells of *Cand*.

Magnetobacterium bavaricum contain between 600 and 1000 magnetosomes that contain bullet-shaped crystals of magnetite and are arranged as several braid-like bundles (usually 3 to 5 per cell) of multiple chains (Hanzlik *et al*., 1996; 2002; Li *et al*., 2010). Because cells of *Cand*. Magnetobacterium bavaricum are mainly found in the microaerobic zone of sediments and contains sulfur-rich globules, it is thought to be a microaerophilic, sulfide-oxidizing bacterium (Spring *et al*., 1993; Jogler *et al*., 2010).

Another magnetotactic *Nitrospirae*, a small rod-shaped bacterium collected from sediment of the Waller See, Germany, was described by Flies and colleagues (2005) and designated strain MHB-1. This organism is a slow-moving, rod-shaped bacterium that contains a single bundle of multiple chains of magnetite magnetosomes whose crystals are also bullet-shaped (Flies *et al*., 2005).

Recently Lefèvre and colleagues (2010) reported the presence of a moderately thermophilic magnetotactic bacterium, designated strain HSMV-1, that belongs to the *Nitrospirae* phylum, in hot springs of the Great Boiling Springs (GBS) geothermal field in Gerlach, Nevada. GBS is a series of hot springs that range from ambient temperature to ~96°C (Anderson, 1978; Costa *et al*., 2009) and those that contained cells of strain HSMV-1 ranged in temperature from 32°C to 63°C. This bacterium, conditionally named *Candidatus* Thermomagnetovibrio paiutensis, is a small vibrio that biomineralizes a single chain of bullet-shaped magnetite magnetosomes.

In a number of freshwater samples collected for magnetotactic bacteria, we noticed in some aquatic sites in south-western USA the presence of unusual, large, ovoid-shaped magnetotactic cells (designated strain LO-1) that are phylogenetically affiliated with the *Nitrospirae* phylum and that have not been previously described. The purpose of this report is to describe and characterize this new bacterium.

Results

Description of sampling sites and samples

Magnetotactic bacteria with similar morphology and size to strain LO-1 were found in water and sediment collected from two sites at Lake Mead including Boulder Beach and Callville Bay (GPS coordinates 36.045664°N, 114.795628°W and 36.141202°N, 114.704862°W respectively); Blue Point Spring (36.389290°N, 114.432961°W); a spring at the Corn Creek Field Station in the Desert National Wildlife Refuge (36.439071°N, 115.359106°W); and a spring in the small town of Blue Diamond (36.046232°N, 115.405846°W) (Fig. S1). All sites are freshwater and with salinities < 1 ppt except Blue

Point Spring which is slightly brackish and with salinity ~3 ppt. Temperature at all sites was ambient except for Blue Point Spring which contains geothermally heated water and was 31°C at the time of sampling. Samples collected from the Lake Mead sites and Blue Point Spring had the highest concentration of LO-1 cells (> 10^4 cells ml^{-1}). These sites had sandy sediments. In the other locations, sediments were muddy and the concentration of LO-1 cells was relatively low (< 10^2 cells ml^{-1}). Water and sediment samples from all sites contained various morpho-types of magnetotactic bacteria including cocci, spirilla, vibrios and rod-shaped cells as well as cells morphologically similar to strain LO-1 (e.g. Fig. 1A; Video S1).

On initial collection, the sediment in samples from Boulder Beach was light brown in colour and there was no odour of hydrogen sulfide. These samples initially contained only magnetotactic cocci and spirilla. After about 5 months of storage in the dark at room temperature, the samples taken at depths 3 and 6 m contained very high numbers of magnetotactic cocci and cells of LO-1 and a relatively small number of magnetotactic spirilla. Enrichment of LO-1 cells did not occur in samples from the other sites. Because cells of LO-1 enriched to high numbers in the samples collected at Boulder Beach, they were the focus of our studies.

Magnetic enrichment and light microscopy of magnetotactic bacteria from samples collected from Lake Mead

Magnetic enrichment of samples by placing the south pole of a magnetic stirring bar next to the sample bottles from Boulder Beach for ~30 min resulted in a visible light brown pellet of magnetotactic bacteria next to the magnet. Even 1 year after collection of these samples, this pellet was clearly visible in the sample collected from 6 m. Cells from the pellet were easily harvested using a Pasteur pipette and light microscopic examination showed the pellet to consist of cells of LO-1, magnetotactic cocci and spirilla (Fig. 1A).

Phylogeny of strain LO-1

Three of seven and one of three 16S rRNA genes cloned and sequenced from magnetic race tracks of magnetically concentrated samples from Boulder Beach and Blue Point Spring, respectively, belonged to the *Nitrospirae* phylum while most of the others belonged to the magnetotactic cocci group in the *Alphaproteobacteria* class. The four sequences belonging to the *Nitrospirae* phylum were similar (> 99.5% identity). We used a *Nitrospirae* oligonucleotide probe specific for *Cand*. Magnetobacterium bavaricum, Mbavp, and fluorescent *in situ* hybridization (FISH) to authenticate the 16S rRNA gene sequence of LO-1. This probe consists of 18 bases, GCCATCCCCTCGCTTACT, has no mismatches to the

rRNA gene sequences of two known magnetotactic *Nitrospirae* (including *Cand*. Magnetobacterium bavaricum and strains MHB-1) and strain LO-1 and thus is a highly effective probe for these organisms. Cells of LO-1 hybridized well to the Mbavp probe while the abundant magnetic cocci, used as a negative control, did not (Fig. 2), indicating that the 16S rRNA gene sequence we retrieved was from strain LO-1.

Based on its 16S rRNA gene sequence, strain LO-1 is not closely related phylogenetically to any other known bacterium (Fig. 3). Its closest relatives are the three other uncultured magnetotactic *Nitrospirae* including the thermophilic vibrio *Candidatus* Thermomagnetovibrio paiutensis (Lefèvre *et al*., 2010) (87.7% identity), the unnamed rod-shaped bacterium strain MHB-1 (Flies *et al*., 2005) (90.1% identity) and *Cand*. Magnetobacterium bavaricum (Spring *et al*., 1993) (91.2% identity). The closest relatives in culture to strain LO-1 are species of the genus *Thermodesulfovibrio* (85.2–85.8% identity).

Distribution of LO-1 cells in natural enrichments

Oxygen and magnetotactic cell concentration profiles were determined using a voltametric microelectrode and light microscopy for the sediment and water sample collected from 6 m depth at Boulder Beach (Fig. 1B). In this sample, there was a broad cell number maximum (peak) of LO-1 cells that started at the oxic–anoxic interface and extended into the top of the anoxic zone. The magneto-tactic cocci were also most concentrated in this same range while the largest numbers of magnetotactic spirilla were located in the anaerobic zone.

Description and ultrastructure of strain LO-1

Cells of strain LO-1 are ovoid in shape and relatively large with an average size of 3.5 ± 0.5 mmby2.7 ± 0.3 mm (n = 53) (Fig. 1A). The majority of cells examined contained inclusions that could be observed using light microscopy (Fig. 4A). Some of these were highly refractile and sulfur-rich as determined by energy-dispersive X-ray spectroscopy analysis (Fig. 4B and C). Cells were Gram-negative and in some cells, two membrane layers representing the inner cytoplasmic membrane and the outer membrane were clearly visible (Fig. 4D). However, a thick amorphous layer close to the external surface of the outer membrane was often present and might represent some sort of capsular material. In other cells, we did not detect clearly defined cytoplasmic and outer membranes but what appeared to be a single three-layered unit membrane layer profile.

Other than magnetosomes, cells appeared to produce two types of inclusions as determined by transmission electron microscopy of thin sections. Both types appeared to

make up the major portion of the cell volume in the cells in which they were present (Fig. 4E and F). The first type was roughly ovoid in shape (Fig. 4E) and relatively large [283 ± 67 by 169 ± 29nm (n = 45)]. Because the contents of these inclusions were easily extracted during preparation for electron microscopy, leaving 'holes' in the thin sections, these likely represent the sulfur-rich globules shown in Fig. 4B. The second type was smaller [151 ± 18 by 115 ± 13 nm(n = 49)] and were spherical to roughly hexagonal in appearance (Fig. 4F). The central part of these inclusions was less electron dense than the peripheral portion. The material in these inclusions was never totally extracted during cell fixation as with the first type of inclusion.

Cells were motile by means of a single polar bundle of flagella that originated from one end of the cell (Fig. 5A). Some of the flagella, if not all, were thicker (~22nm in diameter) than typical unsheathed prokaryotic flagella and had a central core suggestive of the presence of a sheath (Fig. 5B).

Motility of strain LO-1

Cells of strain LO-1 are very motile having an average swimming speed of 116 ± 22 mms $^{-1}$(n = 37). In comparison, the magnetotactic cocci in the same sample had an average swimming speed of 71 ± 16 mms $^{-1}$(n = 61).

When swimming, cells of LO-1 displayed the typical 'wobble' of the bilophotrichous magnetotactic cocci (Video S1). Using long exposure times during photography of swimming cells, we determined that the swimming path of cells of LO-1 is similar to that of the magnetotactic cocci (Frankel *et al*., 1997; Lefèvre *et al*., 2009): cells continually turn while swimming resulting in a twisting, helical pattern during forward swimming (Fig. 5C) and a 'wobble' especially noticeable when cells swim slowly. Cells of LO-1 made about two to four complete rotations during an exposure time of 200 ms (Fig. 3C), a value similar to that of the magnetotactic cocci from Lake Mead which made about three to five rotations in 200 ms (Fig. 5C).

Magnetosomes of strain LO-1

Each cell of strain LO-1 biomineralized approximately 100–200 bullet-shaped magnetosomes arranged as several braid-like bundles (usually three) of multiple chains aligned parallel to the long axis of the cell (Fig. 6A).

These bundles were thick enough in some cells to be observable on occasion using differential interference or phase-contrast light microscopy.

The magnetosomes contained elongated, anisotropic, bullet-shaped crystals that had some differences: the majority of the crystals had one pointed and one flat end while in others, both ends came to a point (Fig. 6B). Both magnetosome crystal types consisted of magnetite as determined by selected area electron diffraction (Fig. 6C). The average size of the magnetosome magnetite crystals with one flat end was 125 _ 22 by 41 ± 3nm (n = 74) while that for those with points at both ends was 137 ± 28 by 45 ± 6nm (n = 71). Thin sections of cells and magnetosomes revealed the presence of an electron-dense layer surrounding and very close to some magnetosome crystals of both types suggestive of a magnetosome membrane (Fig. 6D and E).

Discussion

There are currently few well-described members of the phylum *Nitrospirae* and thus far the group represents a small collection of morphologically and physiologically disparate prokaryotes. Three uncultured *Nitrospirae* are magnetotactic and have been partially characterized (Spring *et al*., 1993; Flies *et al*., 2005; Lefèvre *et al*., 2010). In this report, we characterize a new, fourth magnetotactic member of the group, strain LO-1.

Cells of the freshwater strain LO-1 are large and possess an ovoid cell morphology unlike that of any pre-viously described magnetotactic bacterium. They are mesophilic with regard to temperature. The distribution of LO-1 cells in a natural enrichment was similar to that found for *Cand*. Magnetobacterium bavaricum (Jogler *et al*., 2010): the majority of LO-1 cells were present at the oxic–anoxic interface and the top of the anaerobic zone. These results suggest that LO-1 is either a microaerophile or an anaerobe or both. However, in our attempts at culturing LO-1, we found that cells immediately migrated to the bottom of the tube (anoxic zone) in oxygen-gradient cultures. Cells of LO-1, unlike the magnetotactic cocci from Lake Mead, remained viable the longest (~10 days) in anaerobic enrichments but did not grow. Thus culture experiments indicate that LO-1 is likely an anaerobe that can tolerate low concentrations of oxygen. Interestingly, like cells of *Cand*. Magnetobacterium bavaricum (Jogler *et al*., 2010), many LO-1 cells contained sulfur-rich inclu-sions, the presence of which suggests a metabolism based on the oxidation of reduced sulfur compounds. We did not detect sulfide, the most obvious electron donor for strain LO-1, in our samples (detection limit ~0.1 mM) but this does not preclude its formation (e.g. from sulfatereducing bacteria) as small amounts might be utilized rapidly by sulfide-oxidizing microorganisms and thus would not be detectable.

Magnetotactic bacteria of the LO-1 morphological type appear to be distributed widely in freshwater to brackish environments. Cells with a similar morphology and size as strain LO-1 that we have enriched in this study have been observed and collected from freshwater and estuarine environments including the Exeter River, New Hampshire (Mann *et al*., 1987a,b); the Pettaquamscutt Estuary, Rhode Island (see fig. 3 of Bazylinski and Frankel, 2003); several sites in Germany (fig. 3E of Flies *et al*., 2005; fig. 1D of Amann *et al*., 2006); and freshwater lagoons (Jacarepiá Lagoon, Saquarema, Brazil) (data not shown) and brackish waters (Lagoa de Cima, Rio de Janeiro) in south-eastern Brazil (figs 2.2 and 2.4 of Lins *et al*., 2000).

Cells of LO-1 stain Gram-negative but appear to have an unusual three-layered cell wall. In most cells, the cytoplasmic membrane and the outer membrane were visible and a thick amorphous layer close to the external surface of the outer membrane was present that might represent some type of capsule or polysaccharide layer. In other cells, the cytoplasmic and outer membranes were not clearly defined and the wall seemed to consist of a single trilaminar unit membrane layer. However, this may be due to the oblique sectioning of the cell wall and the relative position of the membrane plane to the incident electron beam under the microscope. This could result in projected images of the membranes within the very thin sections (nominal thickness ~30–50 nm) that show cell wall regions with different numbers of layers.

Cells of LO-1 contain at least two types of intracellular inclusions (excluding magnetosomes). One type appears to be the sulfur-rich bodies or globules discussed above. The other, smaller type is unusual and is a somewhat reminiscent of carboxysomes, an inclusion that contains the CO2-fixing enzyme RubisCO in a number of autotrophic prokaryotes (Yeates *et al*., 2008). Thus far, however, we have not been able to demonstrate autotrophy in LO-1.

Cells of LO-1 are motile and exhibit the rapid swimming velocities and the typical 'wobble' and helical 'flight path' of the bilophotrichous magnetotactic cocci (Sparks *et al*., 1986; Nogueira and Lins de Barros, 1995). This shows that two bundles of flagella are not necessary for the characteristic 'wobble' and helical 'flight path' of the magnetococci and that one flagellar bundle is sufficient. Nogueira and Lins de Barros (1995) obtained the same results with an organism that had similar cell morphology and flagellar arrangement to that of strain LO-1.

Although the cell morphology of strain LO-1 is unique, this organism shares some features in common with the other magnetotactic *Nitrospirae*. For example, cells of strain LO-1, like all other magnetotactic members of the *Nitrospirae*, biomineralize anisotropic, bullet-shaped crystals of magnetite in their magnetosomes (Spring *et al*., 1993; Flies *et al*., 2005; Jogler *et al*., 2010; Lefèvre *et al*., 2010). The only other magnetotactic bacteria known

to biomineralize bullet-shaped magnetite crystals in magnetosomes are phylogenetically affiliated with the *Deltaproteobacteria* class (e.g. *Desulfovibrio magneticus* strain RS-1; Kawaguchi *et al*., 1995; Byrne *et al*., 2010). In LO-1, magnetosomes are arranged as three to four bundles of multiple chains that traverse the cell along its long axis, a situation almost identical to that in cells of *Cand*. Magnetobacterium bavaricum (Jogler *et al*., 2010) and similar organisms (e.g. strain MYR-1; Li *et al*., 2010).

There is some variation in morphology of the magneto-some magnetite crystals in that some crystals have one somewhat flat end and a long pointed end while others have two pointed ends in a two-isosceles triangle with common base motif. Some crystals appear kinked and/or bent, a feature also present in the magnetite crystals of *Cand*. Magnetobacterium bavaricum (Jogler *et al*., 2010). Using high-resolution transmission electron microscopy, Mann and colleagues (1987a,b) examined the morphology and crystal growth of anisotropic bullet-shaped magnetite crystals in an uncharacterized freshwater magnetotactic bacterium having a cell morphology and flagellar pattern very similar to that of strain LO-1. They proposed that the nascent crystals are cuboctahedra which subsequently elongate along [1 1 -2] to form a pseudo-hexagonal prismatic crystal. Biomineralization of this type of magnetite crystal has also been recently studied in the *Cand*. Magnetobacterium bavaricum-like uncultured strain MYR-1 collected from Lake Miyun, China (Li *et al*., 2010). The formation of the bullet-shaped magnetosomes in this organism can also be divided into two stages: initial isotropic growth (to ~20 nm) followed by elongation along the [100] direction (Li *et al*., 2010). Although the [100] orientation is the hard magnetic axis of the face-centred cubic mineral magnetite, the shape anisotropy of the bullet-shaped magnetosomes and intramagnetosome bundle magnetostatic interactions confine the magnetization along the long axis of the magneto-some bundle and therefore the long axis of the cell. Ultimately, each bundle of magnetosome chains effectively behaves as an elongated single-domain particle (Li *et al*., 2010). Based on the similar organization of magnetosomes, it is likely that the situation is the same for *Cand*. Magnetobacterium bavaricum and strain LO-1.

Unlike centrosymmetric magnetite magnetosome crystals (e.g. cubo-octahedra and elongated prisms) of most cultured magnetotactic bacteria (e.g. *Magnetospirillum* species and *Cand*. Magnetococcus marinus), it has recently been shown that bullet-shaped magnetite crystals in the only cultivated strain that has them, *D. magneticus* strain RS-1 (Kawaguchi *et al*., 1995), are not enclosed in a membrane vesicle and lack a magnetosome membrane (Byrne *et al*., 2010). It is thus now important to know whether this is a general phenomenon regarding elongated, anisotropic magnetite particles in bacteria, particularly because the lack of magnetosome membrane might indicate a different mechanism of biomineralization for these crystals than for isotropic magnetite magneto-some crystals. In general, discerning the magnetosome membrane in thin sections of magnetotactic bacteria

is relatively difficult using transmission electron microscopy as recently pointed out by Byrne and colleagues (2010). We examined the magnetosomes of strain LO-1 carefully and found an electron-dense layer surrounding a number of the crystals consistent with the presence of a magnetosome membrane. It did not appear to be the result of 'halo' formation due to underfocusing (Byrne *et al*., 2010). Moreover, although chemical fixation and embedding of the samples can produce more artifacts than cryomicroscopy (Byrne *et al*., 2010), the ultrastructure and thickness of the putative magnetosome membrane in LO-1 are compatible with *Magnetospirillum* cells. To minimize further artifacts and possible misinterpretations, we used very ultra-thin sections (nominal thickness < 40 nm) for imaging the magnetosome membrane and avoided the high underfocus values used in cryomicroscopy samples which are responsible for the halo formation in cryofixed cells. The observation of a magnetosome membrane in LO-1 now raises important questions: does the absence of a magnetosome membrane around bullet-shaped magnetite particles only occur in sulfate-reducing magnetotactic bacteria, or uniquely in *D. magneticus* or in some magnetotactic *Nitrospirae* as well?

Yamazaki and Kawahata (1998) examined a large number of magnetofossils from deep-sea sediments of the Pacific Ocean and showed that isotropic magnetite crystals dominated the magnetofossils in relatively oxidized sediments and anisotropic crystals predominated in more reduced sediments. This suggests that anisotropic magnetite crystals are biomineralized by anaerobic prokaryotes that would be dominant magnetotactic species under reduced conditions such as the sulfate-reducing bacteria. These investigators used these findings to suggest the strong potential of magnetofossil morphology as a paleoenvironmental indicator that could be used as a tool for determining paleo-oxic and anoxic conditions. The fact that strain LO-1 and *Cand*. Magneto-bacterium bavaricum-like strains are found in sediments that are not strongly reducing (this study; Jogler *et al*., 2010) does not support this supposition. Studies involving pure cultures of these organisms where precise conditions under which magnetosome biomineralization occurs can be determined will be necessary to answer this and similar questions.

Based on its phylogeny, strain LO-1 clearly represents a new genus in the *Nitrospirae* phylum in the domain *Bacteria*. As the 16S rRNA gene sequences from LO-1-like cells from both Boulder Beach and Blue Point Springs are virtually identical (> 99.5% identity), it seems that the magnetotactic bacteria observed in our study having the LO-1 morphology from these sites belong to a single species. Based on what we currently know about strain LO-1, we propose the name *Candidatus* Magnetoovum mohavensis (from the Mohave Desert area).

Our results together with the results of Flies and colleagues (2005) and others, and the fact that there are still many unusual, uncultured magnetotactic bacteria that have not

been characterized phylogenetically, suggest that there are more unrecognized magnetotactic members of the *Nitrospirae* in the environment that remain to be discovered.

Experimental procedures

Sampling collection

In this study, water and sediment samples were taken from several different aquatic sites around Las Vegas, Nevada. Lake Mead is the largest reservoir in the USA and was formed by the impoundment of water of the Colorado River by the Hoover Dam. Blue Point Springs is a 'warm spring'; we collected samples in the pool directly below the underground opening of the spring. The water is geothermally heated; however, the source of the water is uncertain. The prevailing theory suggests that the source is located 400 km north in the high mountain ranges near Ely, Nevada. Water from Blue Point Springs feeds into Lake Mead.

Corn Creek is located in the Desert National Wildlife Refuge and is crossed by the Mormon Well Spring. Blue Diamond Spring is located in the small, census-designated town of Blue Diamond west of Las Vegas (Fig. S1). The majority of samples were collected from the shore except for the samples from Lake Mead which were collected by free-diving at depths of 1, 3 and 6 m. One-to two-litre glass or plastic bottles were filled to about 0.2–0.3 of their volume with sediment, the reminder of the bottles filled to their capacity with water that overlaid the sediment. Air bubbles were excluded. Once in the laboratory, samples were stored in the bench at room temperature (~25°C) in the dark or under dim light.

Magnetotactic bacteria with similar morphology to strain LO-1 were observed in most samples over a period of several months. They enriched and reached a concentration $> 10^4$ cells ml^{-1} in some samples from Lake Mead although they became depleted within a month in samples collected from Blue Diamond and Corn Creek Springs.

Light and electron microscopy

The presence and behaviour of microorganisms was observed using light microscopy with a Zeiss (Carl Zeiss MicroImaging, Thornwood, NY) AxioImager M1 light microscope equipped with fluorescence, phase-contrast and differential interference contrast capabilities. The hanging-drop technique (Schüler, 2002) was used routinely in the examination of samples and for quantifying magnetotactic bacteria.

The presence of magnetosomes and the composition of magnetosome crystals and other intracellular inclusions were determined using combinations of electron microscopy, energy dispersive X-ray analysis and selected area electron diffraction with a Tecnai (FEI Company, Hillsboro, OR) Model G2 F30 Super-Twin transmission electron microscope. For ultra-thin sectioning, cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h, washed in the same buffer, dehydrated in an acetone series and embedded in epoxy embedding medium (Fluka Sigma Aldrich GmbH, Steinheim, Switzerland). Ultra-thin sections (nominal thickness ~50 nm) were obtained with a Leica EM U6 ultramicrotome (Leica Microsystems, Bannockburn, IL) stained with uranyl acetate and lead citrate and imaged with a Morgagni transmission electron microscope (FEI Company, Hillsboro, OR).

Chemical and cell count profiles in microcosms

A three microelectrode voltammetric cell was used to determine oxygen and sulfide concentration profiles in a sediment-water sample collected from a 6 m depth at Boulder Beach, Lake Mead. An Ag/AgCl reference electrode and a Pt counter electrode were used in conjunction with an Au/Hg working electrode. Preparation of the solid-state Au/Hg working microelectrode was performed according to Brendel and Luther (1995) and Luther and colleagues (2008). Voltammetric measurements were made with an Analytical Instrument Systems DLK-100 analyser (Analytical Instrument Systems, Flemington, NJ) and recorded to computer. The microelectrode was directed by a micromanipulator. For cell counts, approximately 100 ml of water or water sediment was carefully and slowly removed at specific depths in the sample using a long glass Pasteur pipette with an outer diameter of approximately 1.1 mm and an inner diameter of about 0.8 mm. Cells of LO-1 were counted using the hanging drop technique (Schüler, 2002; Jogler *et al*., 2010) sometimes after the extracted sample was diluted with filter-sterilized water from the sample. Cell counts are reported as the means of triplicate counts from the same depth.

Determination of 16S rRNA gene sequences and phylogenetic analysis

The 16S rRNA gene of magnetically purified cells was amplified using *Bacteria*-specific primers 27F 5′-AGAGTTTGAT CMTGGCTCAG-3′ and 1492R 5′ TACGGHTACCTTGTTAC GACTT-3′ (Lane, 1991). PCR products were cloned into pGEM-T Easy Vector (Promega Corporation, Madison, WI) and sequenced (Functional Biosciences, Madison, WI).

Alignment of 16S rRNA genes was performed using CLUSTAL W multiple alignment accessory application in the BioEdit sequence alignment editor (Hall, 1999). Phylogenetic trees were constructed using MEGA version 4.1 (Tamura *et al*., 2007) applying the neighbour-joining method (Saitou and Nei, 1987). Bootstrap values were calculated with 1000 replicates.

FISH

FISH was used to authenticate the 16S rRNA gene sequence of strain LO-1. Because of the 16S rRNA gene sequence similarity between *Cand*. Magnetobacterium bavaricum and strain LO-1 (at positions 620–637 for *Cand*. Magnetobacterium bavaricum and 632–649 for LO-1), the specific probe Mbavp designed by Spring and colleagues (1993) was used in this study (5′-Alexa 488-GCCATCCCCTCGCTTACT-3′). Hybridization with an Alexa 488-labelled probe was carried out after fixation of magnetically concentrated cells directly on the wells of gelatin-coated hydrophobic microscope slides with 4% paraformaldehyde. FISH was performed according to Pernthaler and colleagues (2001). The hybridization solution contained 10 ng ml^{-1} of the probe, 30% formamide, 0.9 M NaCl, 20 mM Tris-HCl (pH 7.4), 1 mM Na2EDTA and 0.01% sodium deodecyl sulfate (SDS).

Nucleotide sequence accession numbers

16S rRNA gene sequences of the strain LO-1, the magnetic cocci and spirillum from Lake Mead carry GenBank Accession No. GU979422, GU979423 and GU979424 respectively. That from LO-1-like cells from Blue Point Spring is HM466949.

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References

Amann, R., Peplies, J., and Schüler, D. (2006) Diversity and taxonomy of magnetotactic bacteria. In *Magnetoreception and Magnetosomes in Bacteria*, Vol. 3. Schüler, D. (ed.). Berlin, Germany: Springer, pp. 25–36.

Anderson, J.P. (1978) A geochemical study of the southwest part of the Black Rock Desert and its geothermal areas; Washoe, Pershing, and Humboldt counties, Nevada. *Colo School Mines Q* **73:** 15–22.

Bazylinski, D.A., and Frankel, R.B. (2003) Biologically controlled mineralization in prokaryotes. *Rev Miner Geochem* **54:** 217–247.

Bazylinski, D.A., and Frankel, R.B. (2004) Magnetosome formation in prokaryotes. *Nat Rev Microbiol* **2:** 217–230.

Brendel, P.J., and Luther, G.W., III, (1995) Development of a gold amalgam voltammetric microelectrode for the determination of dissolved Fe, Mn, O2, and S(-II) in porewaters of marine and fresh-water sediments. *Environ Sci Technol* **29:** 751–761.

Byrne, M.E., Ball, D.A., Guerquin-Kernc, J.-L., Rouillere, I., Wuc, T.-D., Downing, K.H., *et al*. (2010) *Desulfovibrio magneticus* RS-1 contains an iron-and phosphorus-rich organelle distinct from its bullet-shaped magnetosomes. *Proc Natl Acad Sci USA* **107:** 12263–12268.

Costa, K.C., Navarro, J.B., Shock, E.L., Zhang, C.L., Soukup, D., and Hedlund, B.P. (2009) Microbiology and geochemistry of great boiling and mud hot springs in the United States Great Basin. *Extremophiles* **13:** 447–459.

Ehrich, S., Behrens, D., Lebedeva, E., Ludwig, W., and Bock, E. (1995) A new obligately chemolithoautotrophic, nitrite-oxidizing bacterium, *Nitrospira moscoviensis* sp. nov. and its phylogenetic relationship. *Arch Microbiol* **164:** 16–23.

Flies, C.B., Peplies, J., and Schüler, D. (2005) Combined approach for characterization of uncultivated magnetotactic bacteria from various aquatic environments. *Appl Environ Microbiol* **71:** 2723–2731.

Frankel, R.B., Bazylinski, D.A., Johnson, M.S., and Taylor, B.L. (1997) Magneto-aerotaxis in marine coccoid bacteria. *Biophys J* **73:** 994–1000.

Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/ NY. *Nucleic Acids Symp Ser* **41:** 95–98.

Hanzlik, M., Winklhofer, M., and Petersen, N. (1996) Spatial arrangement of chains of magnetosomes in magnetotactic bacteria. *Earth Planet Sci Lett* **145:** 125–134.

Hanzlik, M., Winklhofer, M., and Petersen, N. (2002) Pulsedfield-remanence measurements on individual magnetotactic bacteria. *J Magn Magn Mater* **248:** 258–267.

Haouari, O., Fardeau, M.-L., Cayol, J.-L., Fauque, G., Casiot, C., Elbaz-Poulichet, F., *et al*. (2008) *Thermodesulfovibrio hydrogeniphilus* sp. nov., a new thermophilic sulphate-reducing bacterium isolated from a Tunisian hot spring. *Syst Appl Microbiol* **31:** 38–42.

Henry, E.A., Devereux, R., Maki, J.S., Gilmour, C.C., Woese, C.R., Mandelco, L., *et al*. (1994) Characterization of a new thermophilic sulfate-reducing bacterium *Thermodesulfovibrio yellowstonii*, gen. nov. and sp. nov.: its phylogenetic relationship to *Thermodesulfobacterium commune* and their origins deep within the bacterial domain. *Arch Microbiol* **161:** 62–69.

Hippe, H. (2000) *Leptospirillum* gen. nov. (*ex* Markosyan 1972), nom. rev., including *Leptospirillum ferrooxidans* sp. nov. (*ex* Markosyan 1972), nom. rev. and *Leptospirillum thermoferrooxidans* sp. nov. (Golovacheva *et al*. 1992). *Int J Syst Bacteriol* **50:** 501–503.

Jogler, C., Niebler, M., Lin, W., Kube, M., Wanner, G., Kolinko, S., *et al*. (2010) Cultivation-independent characterization of '*Candidatus* Magnetobacterium bavaricum' via ultrastructural, geochemical, ecological and metagenomic methods. *Environ Microbiol* **12:** 2466–2478.

Kawaguchi, R., Burgess, J.G., Sakaguchi, T., Takeyama, H., Thornhill, R.H., and Matsunaga, T. (1995) Phylogenetic analysis of a novel sulfate-reducing magnetic bacterium, RS-1, demonstrates its membership of the d-Proteobacteria. *FEMS Microbiol Lett* **126:** 277–282.

Lane, D.J. (1991) 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*. Stackebrandt, E., and Goodfellow, M. (eds). Chichester, UK: Wiley & Sons, pp. 115–175.

Lefèvre, C.T., Bernadac, A., Yu-Zhang, K., Pradel, N., and Wu, L. (2009) Isolation and characterization of a magneto-tactic bacteria from the Mediterranean Sea. *Environ Microbiol* **11:** 1646–1657.

Lefèvre, C.T., Abreu, F., Schmidt, M.L., Lins, U., Frankel, R.B., Hedlund, B.P., and Bazylinski, D.A. (2010) Moderately thermophilic magnetotactic bacteria from hot springs in Nevada. *Appl Environ Microbiol* **76:** 3740– 3743.

Li, J., Pan, Y., Liu, Q., Yu-Zhang, K., Menguy, N., Che, R., *et al*. (2010) Biomineralization, crystallography and magnetic properties of bullet-shaped magnetite magnetosomes in giant rod magnetotactic bacteria. *Earth Planet Sci Lett* **293:** 368–376.

Lins, U., Freitas, F., Keim, C.N., and Farina, M. (2000) Electron spectroscopic imaging of magnetotactic bacteria: magnetosome morphology and diversity. *Microsc Microanal* **6:** 463–470.

Luther, G.W., III, Glazer, B.T., Ma, S., Trouwborst, R.E., Moore, T.S., Metzger, E., *et al*. (2008) Use of voltammetric solid-state (micro)electrodes for studying biogeochemical processes: laboratory measurements to real time measurements with an *in situ* electrochemical analyzer (ISEA). *Mar Chem* **108:** 221–235.

Mann, S., Sparks, N.H.C., and Blakemore, R.P. (1987a) Ultrastructure and characterization of anisotropic magnetic inclusions in magnetotactic bacteria. *Proc R Soc Lond B* **231:** 469–476.

Mann, S., Sparks, N.H.C., and Blakemore, R.P. (1987b) Structure, morphology and crystal growth of anisotropic magnetite crystals in magnetotactic bacteria. *Proc R Soc Lond B* **231:** 477–487.

Nogueira, F.S., and Lins de Barros, H.G.P. (1995) Study of the motion of magnetotactic bacteria. *Eur Biophys J* **24:** 13–21.

Pernthaler, J., Glöckner, F.O., Schönhuber, W., and Amann, R. (2001) Fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes. *Methods Microbiol* **30:** 207–226.

Petersen, N., Weiss, D.G., and Vali, H. (1989) Magnetic bacteria in lake sediments. In *Geomagnetism and Paleomagnetism*. Lowes, F.J., Collinson, D.W., Parry, J.H., Runcorn, S.K., Tozer, D.C., and Soward, A. (eds). Dordrecht, the Netherlands: Kluwer Academic Publishers, pp. 231– 241.

Saitou, N., and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4:** 406–425.

Schübbe, S., Williams, T.J., Xie, G., Kiss, H.E., Brettin, T.S., Martinez, D., *et al*. (2009) Complete genome sequence of the chemolithoautotrophic marine magnetotactic coccus strain MC-1. *Appl Environ Microbiol* **75:** 4835–4852.

Schüler, D. (2002) The biomineralization of magnetosomes in *Magnetospirillum gryphiswaldense*. *Int Microbiol* **5:** 209– 214.

Sekiguchi, Y., Muramatsu, M., Imachi, H., Narihiro, T., Ohashi, A., Harada, H., *et al*. (2008) *Thermodesulfovibrio aggregans* sp. nov. and *Thermodesulfovibrio thiophilus* sp. nov., anaerobic, thermophilic, sulfate-reducing bacteria isolated from thermophilic methanogenic sludge, and emended description of the genus *Thermodesulfovibrio*. *Int J Syst Bacteriol* **58:** 2541–2548.

Sonne-Hansen, J., and Ahring, B.K. (1999) *Thermodesulfobacterium hveragerdense* sp. nov., and *Thermodesulfovibrio islandicus* sp. nov., two thermophilic sulfate reducing bacteria isolated from a Icelandic hot spring. *Syst Appl Microbiol* **22:** 559–564.

Sparks, N.H.C., Courtaux, L., Mann, S., and Board, R.G. (1986) Magnetotactic bacteria are widely distributed in sediments in the U.K. *FEMS Microbiol Lett* **37:** 305– 308.

Spring, S., Amann, R., Ludwig, W., Schleifer, K.-H., van Gemerden, H., and Petersen, N. (1993) Dominating role of an unusual magnetotactic bacterium in the microaerobic zone of a freshwater sediment. *Appl Environ Microbiol* **50:** 2397–2403.

Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24:** 1596–1599.

Vali, H., Forster, O., Amarantidid, G., and Petersen, H. (1987) Magnetotactic bacteria and their magnetofossils in sediments. *Earth Planet Sci Lett* **86:** 389–400.

Watson, S.W., Bock, E., Valois, F.W., Waterbury, J.B., and Schlosser, U. (1986) *Nitrospira marina* gen. nov. sp. nov.: a chemolithotrophic nitrite-oxidizing bacterium. *Arch Microbiol* **144:** 1–7.

Yamazaki, T., and Kawahata, H. (1998) Organic carbon flux controls the morphology of magnetofossils in marine sediments. *Geology* **26:** 1064–1066.

Yeates, T.O., Kerfeld, C.A., Heinhorst, S., Cannon, G.C., and Shively, J.M. (2008) Protein-based organelles in bacteria: carboxysomes and related microcompartments. *Nat Rev Microbiol* **6:** 681–691.

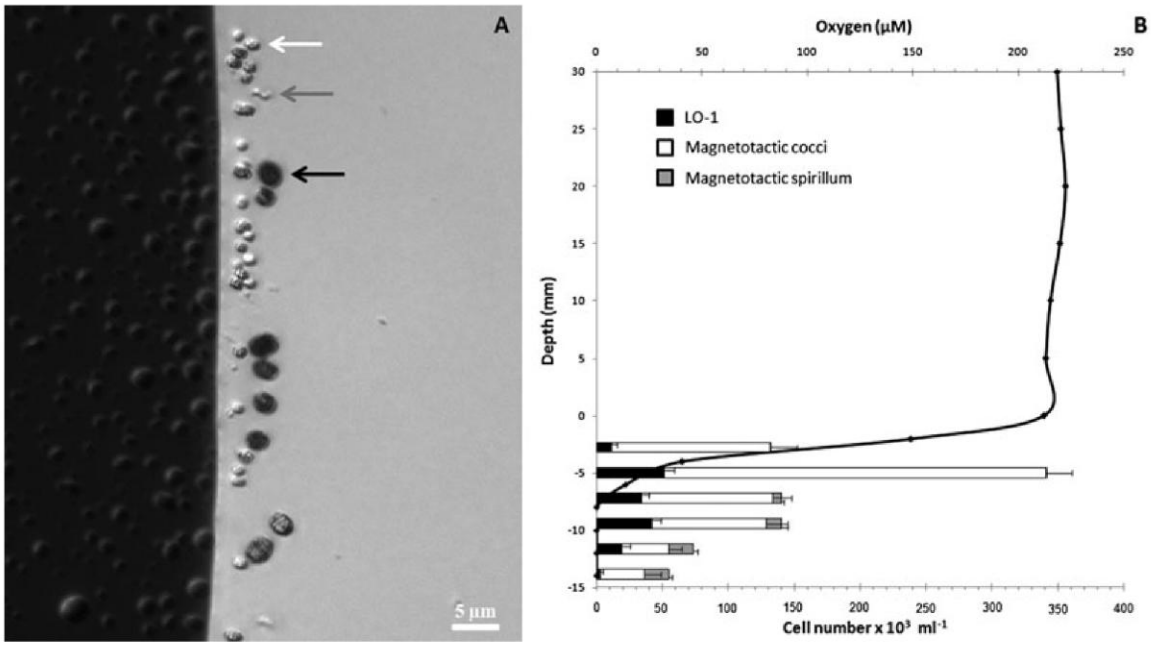

Fig. 1. A. Differential interface contrast (DIC) light micrograph of north-seeking magnetotactic bacteria from a magnetic enrichment of magnetotactic bacteria from a sample collected from Boulder Beach at Lake Mead using the hanging drop technique. Note the presence of magnetotactic cocci (at white arrow) and spirilla (at grey arrow) and the large ovoid cells of strain LO-1 (at black arrow).
B. Vertical concentration profiles of oxygen and specific magnetotactic bacterial morphotypes through the water column and surface sediments of a bottled sample (microcosm) collected at 6 m depth at Boulder Beach, Lake Mead. The microcosm had been incubated in the dark at room temperature for approximately 13 months prior to taking profile measurements. Note the measurements extend through the oxic–anoxic interface and the upper regions of the anaerobic zone of the sediment. Cell counts are reported as the mean of triplicate measurements and line extensions represent the positive standard deviation.

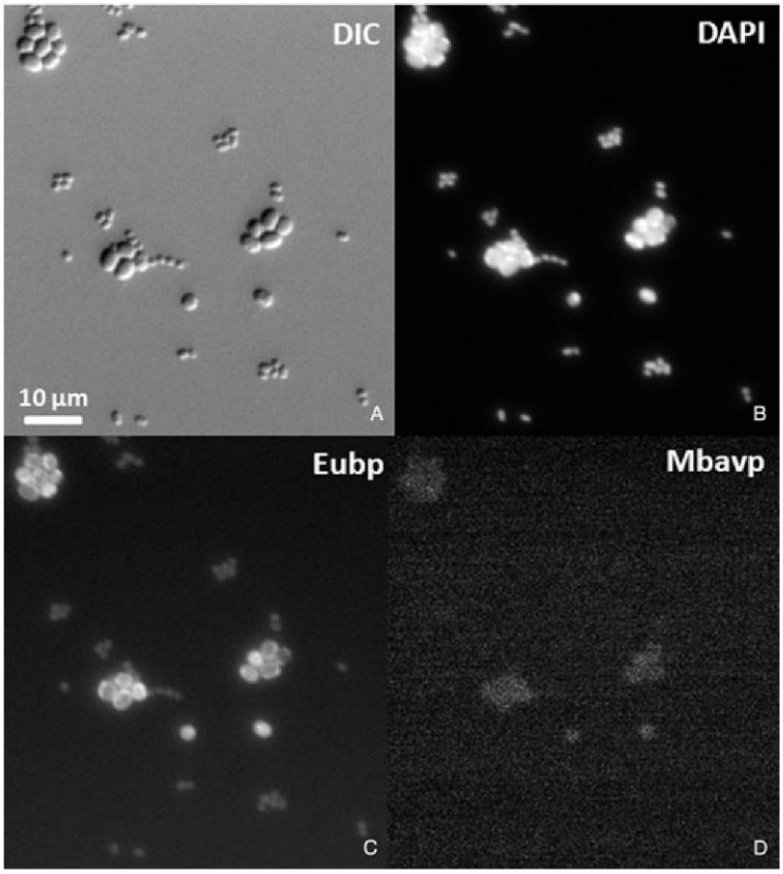

Fig. 2. Fluorescent *in situ* hybridization (FISH) of cells of strain LO-1 using the specific oligonucleotide rRNA probe (Mbavp) originally designed for *Candidatus* Magnetobacterium bavaricum (Spring *et al.*, 1993).
A. Differential interference contrast (DIC) image of strain LO-1 (largest cells) and magnetotactic cocci (smaller cells use as negative control) magnetically enriched from samples.
B. Fluorescence microscope image of the same cells stained with 4′,6-diamidino-2-phenylindole (DAPI).
C. Fluorescence microscope image of the same cells hybridized with the Bacteria-specific probe Eubp. Note both LO-1 cells and the magnetotactic cocci fluoresce with this probe although with less intensity.
D. Fluorescence microscope image of the same cells hybridized with the *Candidatus* Magnetobacterium bavaricum-specific probe Mbavp. Note only LO-1 cells fluoresce with this probe.

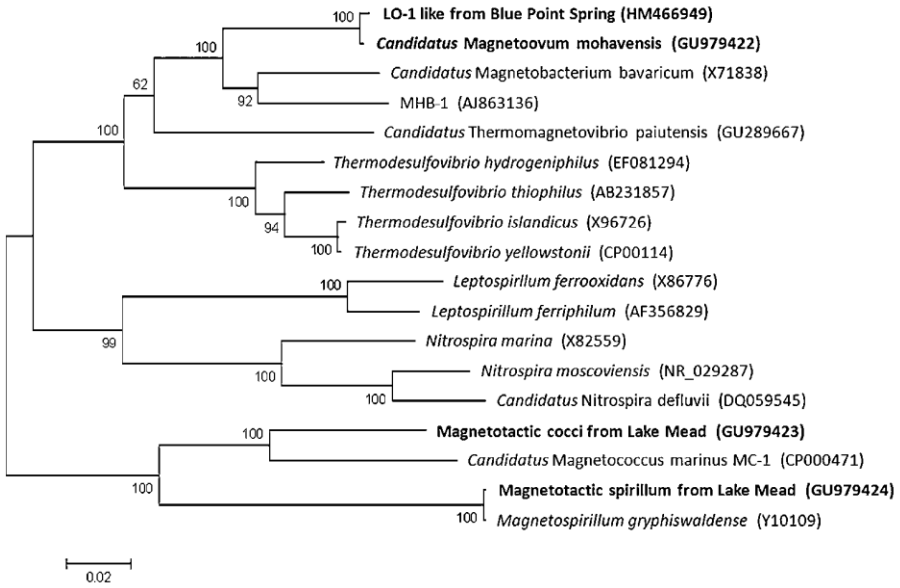

Fig. 3. Phylogenetic tree, based on 16S rRNA gene sequences, showing the phylogenetic position of strain LO-1 in the phylum *Nitrospirae*. Bootstrap values at nodes are percentages of 1000 replicates. The magnetotactic bacteria *Candidatus* Magnetococcus marinus (strain MC-1; Schübbe *et al.*, 2009), *Magnetospirillum gryphiswaldense* strain MSR-1 and the magnetotactic cocci and spirillum from Lake Mead (outgroup; *Alphaproteobacteria* class) were used to root the tree. GenBank accession numbers are given in parentheses. Bar represents 2% sequence divergence.

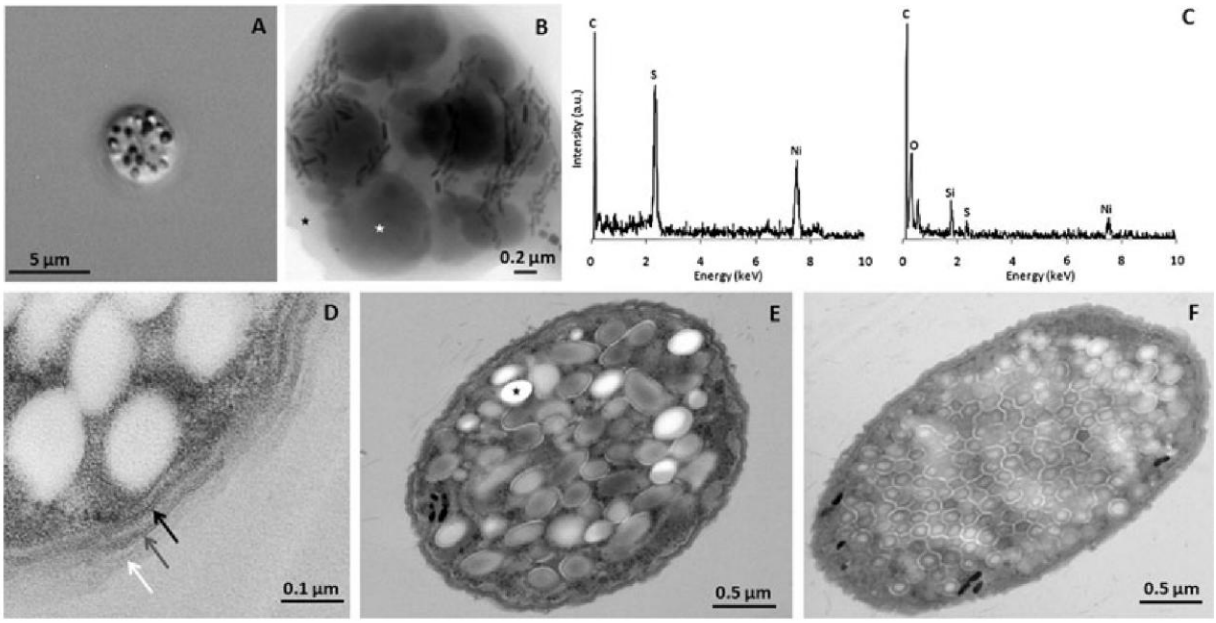

Fig. 4. Ultrastructure of cells of strain LO-1.
A. DIC light microscope image showing the numerous, large, highly refractile, intracellular inclusions within a cell of strain LO-1.
B. Transmission electron microscope (TEM) image of an unstained LO-1 cell showing large globular inclusions and magnetosomes.
C. Elemental spectra of an inclusion (beam focused at white star) and background of the cell (beam focused at black star) using energy dispersive X-ray spectroscopy analysis. Note that the globular inclusion is sulfur-rich and appears to be similar to the type of sulfur-containing inclusions typical of sulfide-oxidizing bacteria.
D. TEM image of a stained thin section of a cell of LO-1 showing the complex tripartite cell wall composed of the cytoplasmic membrane (at black arrow), the outer membrane (at grey arrow) and the external amorphous layer (at white arrow). The latter might represent some type of polysaccharide layer. The 'empty' inclusions appear to be the same shown in (F).
E and F. TEM images of a thin section of a stained LO-1 cells showing the two types of numerous inclusions present in LO-1 cells. Those in (E) show some degree of extraction during fixation [shown as 'holes' (at star)] and could be the sulfur-rich inclusions described above. Note the smaller inclusions shown in (F) have an electron-dense periphery with a less dense centre.

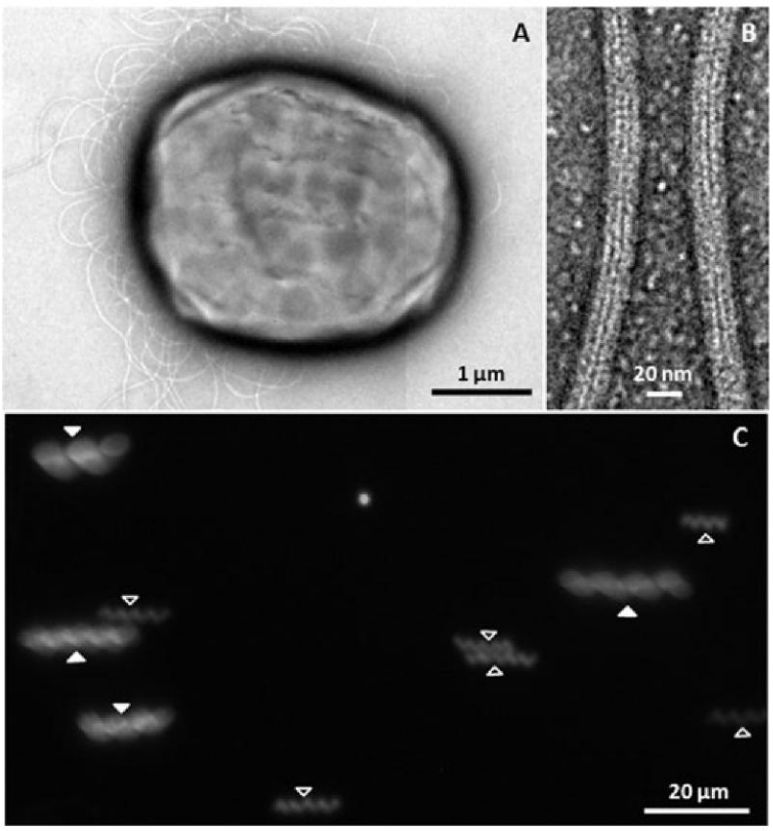

Fig. 5. Flagella and motility of strain LO-1.
A. TEM image of a negatively stained LO-1 cell showing the presence of a single polar bundle of flagella.
B. High-magnification TEM of individual flagella. Note that flagella show a central core and are thicker (~22 nm in diameter) than typical prokaryotic flagella. Both features indicate that the flagella are sheathed.
C. Dark-field light microscope image using a long 200 ms exposure time demonstrating the helical pattern of motility during forward swimming, magnetically directed, by both the magnetotactic cocci (empty arrowheads) and strain LO-1 (filled arrowheads) collected from Boulder Beach, Lake Mead. Note that cells of LO-1 make about two to four rotations during the exposure time of 200 ms, a value similar to that of the magnetotactic cocci which make about three to five rotations in 200 ms.

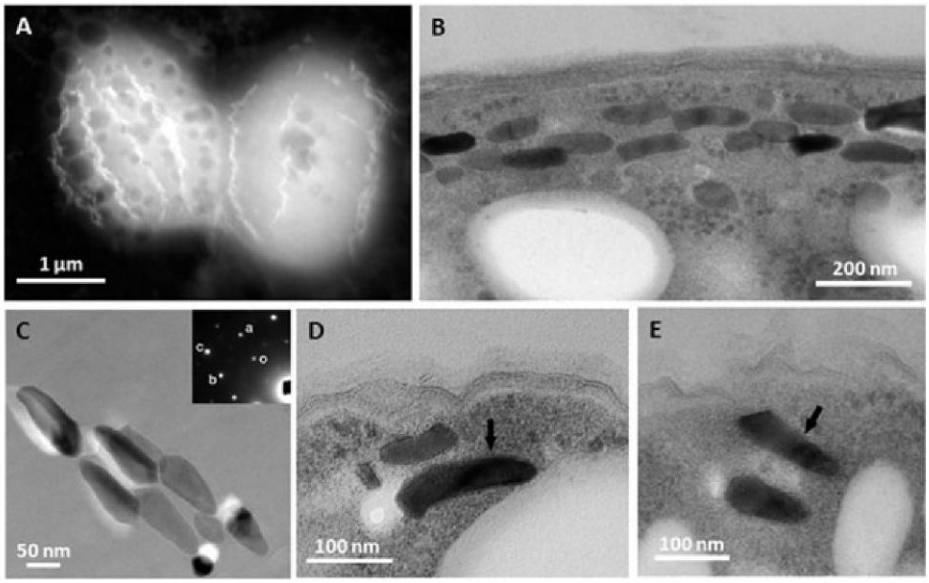

Fig. 6. Magnetosome organization and magnetosome crystal morphology and composition in LO-1 cells.
A. Scanning-transmission electron microscope (STEM) image showing organization of magnetosomes as three or four bundles of chains parallel to the long axis of the cell.
B–E. TEM image of a stained thin section of an LO-1 cells showing two types of anisotropic bullet-shaped magnetosome crystals within the chain bundle. One type has one pointed and one flat end while in the other, both ends came to a point, one longer than the other. (C) TEM image of magnetosomes within a cell of LO-1. Inset shows selected area electron diffraction pattern from magnetosomes shown in (C). The pattern corresponds to the [1 −1 0] zone of magnetite, Fe_3O_4: reflection o, (000); reflection a, (002) (0.40 nm); reflection b, (220) (0.29 nm); reflection c, (222) (0.22 nm); angle a-o-b, 90°; angle b-o-c, 35°. (D and E) High-magnification TEM images of stained thin sections of magnetosomes within cells of LO-1. Note the presence of an electron-dense layer surrounding both types of anisotropic magnetite crystals suggestive of the presence of a magnetosome membrane.